

The University of Chicago

I. THE COMPARATIVE ASSAY OF
GONADOTROPIC SUBSTANCES ON RATS,
MICE, AND CHICKS

II. COMPARATIVE STUDY ON THE
AUGMENTATION OF THE ACTION OF
GONADOTROPINS FROM VARIOUS SOURCES
IN THE RAT, MOUSE, AND CHICK

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

1939

By

JOHN STONE EVANS

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THE COMPARATIVE ASSAY OF GONADOTROPIC SUBSTANCES ON RATS, MICE AND CHICKS^{1,2}

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ONE OF THE MAJOR PROBLEMS in the fractionation of gonadotropic substances is the development of suitable assay methods. The literature contains numerous methods developed by different workers but one is unable to convert the proposed units of one worker into the units of another because of the different methods used and the different types of preparations studied. The purpose of this study is to compare the results obtained from the assay of gonadotropic preparations from sheep pituitary glands, normal male urine, menopause urine and pregnant mare serum on the immature female rat, the immature female mouse, and the 1-day-old chick.

MATERIALS AND METHODS

The anterior pituitary preparation used in this study was prepared by suspending the powdered, acetone-desiccated glands in 0.25% ammonium hydroxide for 24 hours. The pH of the extracting medium was then adjusted to 5.0 to 5.6, and the gland residue centrifuged off. The gland residue was re-extracted with an acetic acid buffer (pH 5.6). The combined extracts were mixed with 3 volumes of 95% ethyl alcohol and placed in the freezer (-3°C) for 24 hours. The resulting precipitate was centrifuged off and dried with either absolute alcohol or acetone and then ether. The dried precipitate is referred to as "pituitary powder A" in this paper.

Gonadogen, a preparation from pregnant mare serum, was supplied by Dr. George Cartland of The Upjohn Company. The assays on this material are expressed in terms of the manufacturer's units. Cartland and Nelson (1) define their unit "as the minimum total dose of hormone which, administered to 21- to 23-day-old rats weighing 35 to 45 gm. in 3 equal subcutaneous injections at daily intervals, will produce at autopsy, 96 hours after the first injection, a mean ovarian weight of 65 mg. which is 4 to 5 times that of the controls."

Prospermin, prepared from normal male urine, and gamone, from menopause urine, were supplied by Dr. J. A. Morrell, E. R. Squibb & Sons Company. The units used in the assays on these substances are reported in terms of the manufacturer's units. Morrell (2) defines a Squibb unit as the total amount of material which, when injected into a group of 29- to 30-day-old immature female rats for 3 days will cause a uterine response in 50% of the animals. A standard powder is run in parallel. Morrell states that a Squibb unit is equivalent to 5 Levin-Tyndale mouse units.

The rat assays reported in this paper were run by the method of Wallen-Law-

¹ These investigations were supported in part by grants from Armour and Company and the Otho S. A. Sprague Memorial Institute.

² We acknowledge our thanks to Dr. Edwin F. Pike of Armour & Company for the fresh pituitary glands, Dr. George F. Cartland of the Upjohn Company for the gonadogen, and Dr. J. A. Morrell of Squibb & Sons for the prospermin and gamone.

rence and Van Dyke (3). Twenty-one-day-old female rats were injected subcutaneously once daily for 4 days and, 48 hours after the final injection, the ovaries and uteri were freed from any adhering mesentery and weighed on a Roller-Smith torsion balance.

The mouse assays were conducted by a method similar to that used by Hamburger and Pedersen-Bjergaard (4). Female mice, weighing 6 to 8 gm. were injected subcutaneously twice daily for 3 days, and 48 hours after the final injection, the ovaries and uteri were freed from any adhering tissues and weighed on the torsion balance.

The chick assay was developed in this laboratory and was conducted as follows.

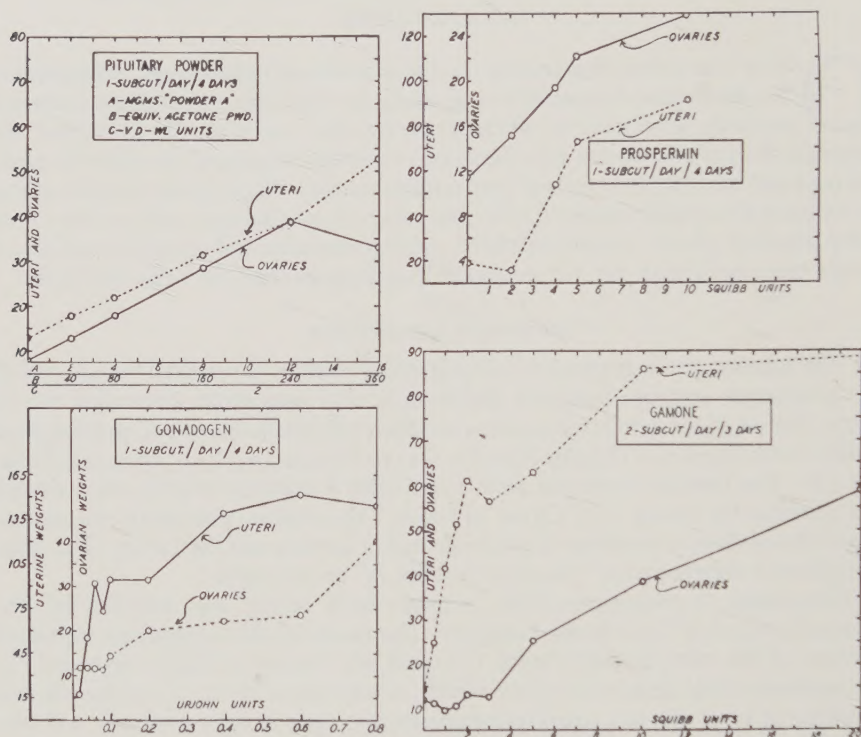


Fig. 1. GONADOTROPIC ASSAYS ON THE RAT WITH GLANDULAR, URINARY AND BLOOD SERUM PREPARATIONS. Animals killed 48 hours after last injection.

One-day old cockerels were divided into groups of 20 each. Each group was injected twice daily for 5 days. Injections were made subcutaneously in the front of the legs, alternating the sides at each injection. Care was taken to use a small needle to avoid leakage. Eighteen hours after the final injection, the testes and the thyroids were weighed on the torsion balance.

EXPERIMENTAL RESULTS

The results of the rat assays are given in figure 1. The ovaries of the rats receiving the pituitary preparations were filled with corpora lutea. It is to be noted that the rat uterus is not a more sensitive indicator than the ovary to the glandular preparation. The uteri were mostly thick-walled with a few of the thin-walled estrous type. The preparation used was unfractionated and the relative amounts of FSH and LH no

doubt play an important part in the amount of the mixture required to produce a uterine response. Pregnant mare serum, normal male urine, and menopause urine preparations which have been shown to be follicle-stimulating substances, produced follicular stimulation at low levels and luteinization at the higher levels. In the case of the last three products, the rat uteri are much more sensitive indicators than the ovary. It should be noted that per Squibb unit, gamone at low levels is very much more active as measured by uterine weight than prospermin. At higher levels the differences are not so striking.

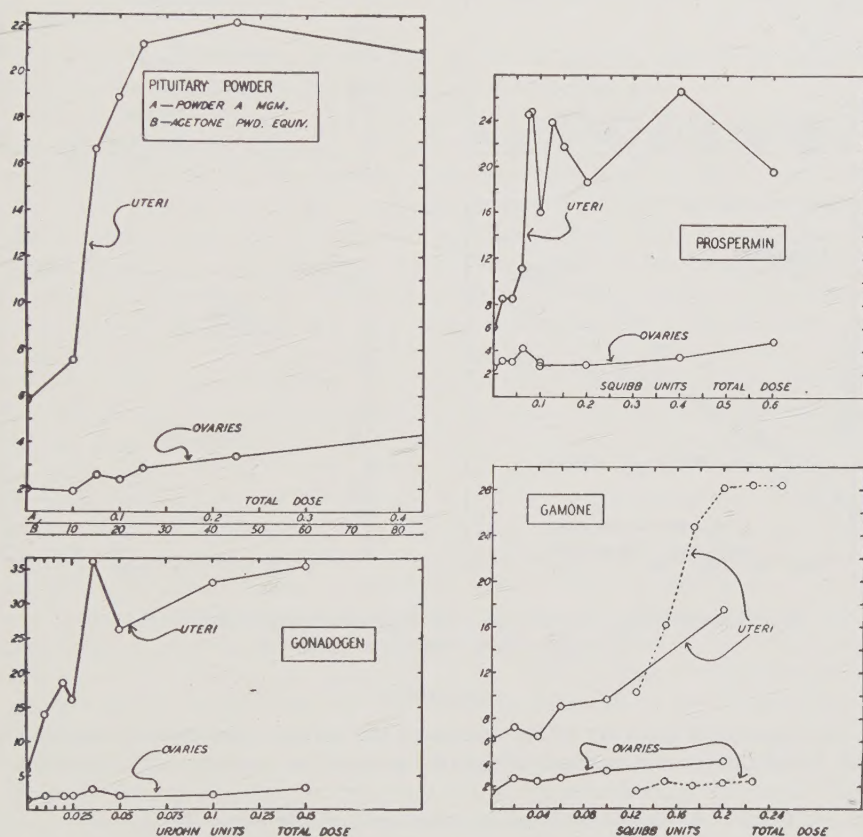


Fig. 2. GONADOTROPIC ASSAYS ON THE MOUSE. Two subcutaneous injections per day for 3 days, organs weighed 48 hours later.

The results of the mouse assays are shown in figure 2. In the mouse, the uterus is a very much more sensitive indicator than the ovary to all the preparations with the possible exception of castrate or menopause urine (gamone).

The results of the chick assays are shown in figure 3. The testes of the chick respond to the injection of pituitary extracts, pituitary suspensions, and pregnant mare serum, but not to pregnancy urine, menopause urine (gamone), and normal male urine (prospermin) preparations. The thyrotropic activity of the pituitary extracts can be assayed at the same time. The pregnant mare serum preparation showed no thyrotropic activity. The left gonad of the 1-day-old Leghorn pullet was found to be less sensitive to pituitary extracts than the testes of the 1-day-old Leghorn cockerel.

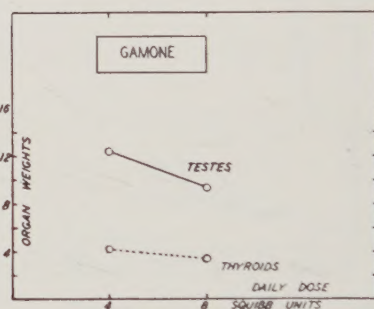
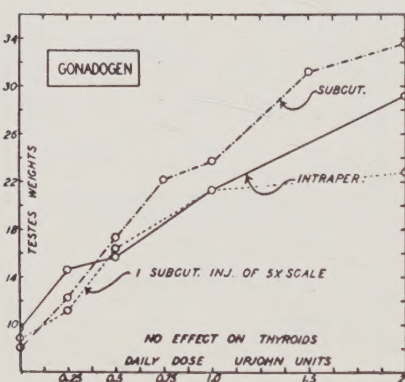
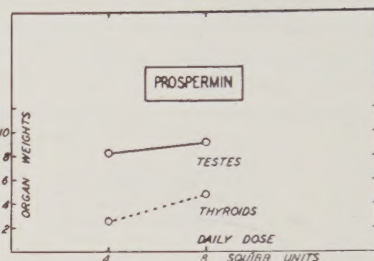
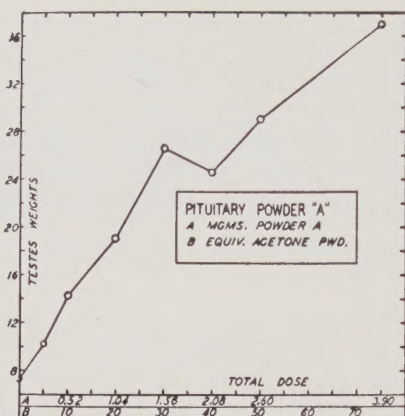


Fig. 3. GONADOTROPIC ASSAYS ON THE CHICK. Two subcutaneous injections per day for 5 days, organs weighed on the 6th day.

DISCUSSION

A comparative summary of our results by the various assay methods is given in table 1 and the relative amounts of material required to produce 100% increases in

TABLE 1. A COMPARATIVE SUMMARY OF THE RESULTS FROM GONADOTROPIC ASSAYS

	Species and organs	Pituitary powder A	Mare serum, gonadogen	Male urine, prosermin	Menopause urine, gamone
		mg.	Upjohn u.	Squibb u.	Squibb u.
Minimum doses to produce 100% increases in organ weights	Rat uterus	5.3	0.03	3.5	0.75
	Rat ovary	4	0.6	6.0	4.25
	Mouse uterus	0.06	0.01	0.065	0.13
	Mouse ovary	0.35	0.15	0.6	0.1
	Chick testis	0.65	0.9	8	toxic
Organ and species ratios for the minimum doses	Rat O/Rat U	0.75	20	1.7	5.6
	Mouse O/Mouse U	5.9	Ca 15	9.2	0.8
	Rat O/Mouse O	11	4	10	42
	Rat U/Mouse U	88	3	50	6
	Chick T/Mouse U	10	90	120	—

TABLE 2. RELATIVE AMOUNT OF GONADOTROPIC SUBSTANCE REQUIRED BY EACH SPECIES FOR THE 100% INCREASE IN ORGAN WEIGHTS

Gonadotropin preparation	Rat ovary	Rat uterus	Chick testis	Mouse uterus
Pituitary powder A	66	88	11	1
Pregnant mare serum, gonadogen	60	3	90	1
Male urine, prospermin	92	54	123?	1
Menopause urine, gamone	33	5.8	toxic	1

The absolute amounts required will depend upon the assay method used. The values given are for the particular methods described.

organ weights are given in table 2. The amount of material required to produce a 100% increase in the weight of the mouse uterus is taken as unity.

Examination of figures 1, 2 and 3 and table 1 reveals that the 4 gonadotropins act very differently quantitatively in the 3 species and even in the same species depending on the organ weight used as a measure. Even the 2 urine preparations act very differently on the Squibb unit basis. To illustrate more specifically, it requires approximately 4 times as many Squibb units of prospermin as of gamone to produce the 100% increase in the rat uterus and only 1.4 times the amount for the same change in the rat ovary. In the mouse the orders are reversed. Here only one-half the number of Squibb units of prospermin as of gamone are required for the 100% increase in the mouse uterus, but 6 times the amount for the same result in the mouse ovary. In the chick the testis response requires very high doses of prospermin and the gamone is too toxic to use. The results with pituitary powder A and gonadogen are equally irregular. Thus, the ratio of mg. of powder A to Upjohn units of gonadogen to produce the 100% weight increase of the rat uterus is 176, but for the rat ovary it is 6.6, for the mouse uterus, 6, for the mouse ovary, 2, and for the chick testis, 0.7. Comparison of the ratio of mg. of powder A to Squibb units of prospermin comes the nearest to a consistent agreement in the different species. Thus, for rat uterus it is 1.5, for rat ovary, 0.66, for mouse uterus, 1.0, for mouse ovary, 0.6, and for chick testis, 0.08. The only consistent finding obvious from tables 1 and 2 is the high sensitivity of the immature mouse uterus to the 4 types of the gonadotropins investigated. Inspection of table 2 reveals a rather close agreement in the relative amounts of gonadogen and gamone required for the mouse and rat uterine responses and a much higher ratio for the relative amounts of powder A and prospermin.

These different responses cannot be explained satisfactorily at the present time. The main difficulty is that we are using impure gonadotropin preparations. All of them may contain true follicle stimulating substances, some luteinizing material, interstitial cell-stimulating material, augmenting as well as inhibiting factors and other inert or toxic substances in different proportions. Whether the true follicle-stimulating substances or other gonadotropic factors are identical in any of the preparations, and to what extent we have varying amounts of luteinizing material present in the preparations, or how much we are complicating the assay methods by not eliminating the test animal's own pituitary secretions by using hypophysectomized animals remains to be determined.

A direct comparison of our results with those of others is more difficult because slight differences in the details of the assay methods, different methods of preparation, differences in the age and origin of the test animals, and other variables complicate the problem still more. Brief consideration of the main contributions by others to this comparative aspect of the pituitary problem, however, shows close agreement with our general conclusions on the relative merits of the different methods of assays.

Rat Ovarian and Uterine Responses

It is now generally agreed that the rat uterine response to gonadotropins is much more sensitive than the rat ovarian response. The former is primarily an FSH response, the latter may represent an FSH plus LH response. Heller et al. (5, 6) found that the rat uterus is 8 times as sensitive as the ovary toward crude suspensions of castrate rat pituitaries. They also reported a greater sensitivity toward urinary preparations. Cartland and Nelson (1) with pregnant mare serum preparations reported a 10- to 15-fold sensitivity for the rat uterine response, but considered the ovarian weight increase a better quantitative response. They state that the uterine response is a very sensitive qualitative test and that it represents primarily the FSH factor. With higher doses of their material they always observed a greater ovarian response and luteinization. D'Amour and D'Amour (7) from a comparative study on the assay of gonadotropins from pregnancy urine, placenta and sheep pituitaries concluded that the immature male rat is more sensitive to the first two, that corpus luteum formation was a matter of high enough dosage, that the opening of the vagina was a very unreliable criterion, that the positive estrous vaginal smear was very sensitive for urinary preparations, and that ovarian weight was the "most objective" criterion although at times a marked ovarian response resulting from glandular preparations gave negative estrous smears. Unfortunately, the authors did not weigh the uteri. Cohen and Ardoint (8) also agree that the rat uterine response is a very sensitive indicator.

However, Lauson et al. (9) claim that a greater rat uterine response to pituitary FSH is obtained if the LH factor is added also. Although they claim that a uterine response cannot be considered a reliable measure of FSH concentration, they, nevertheless, recommend the uterine response as a very sensitive test. No doubt the difficulty of separating the FSH and LH factors satisfactorily for assay purposes and the elimination of the factor or factors introduced by the test animal's own pituitary are the main difficulties involved in routine assays. The ideal method is to use only hypophysectomized animals for assay purposes.

The Mouse Assay

Levin and Tyndale (10) studied the application of the mouse uterus to the assay of gonadotropin preparations from castrate urine, normal male urine, pregnancy urine, pregnant mare serum and FSH from sheep pituitaries and concluded that male and castrate urine preparations give essentially the same quantitative response, that the glandular preparation gives a flatter curve, and that the method is not as accurate for the chorionic substances, pregnancy urine extracts, and pregnant mare serum, as for castrate urine. Hamburger and Pedersen-Bjergaard (4) studied the mouse and rat assay of pregnancy urine and pregnant mare serum. They concluded that the ovarian weight response is the best method for standardizing the pregnant mare serum and that the rat gives the most consistent results, but that the vaginal smear of immature rats or the determination of corpora lutea in the ovaries of mice are the best suited for the assay of the gonadotropic agent from pregnancy urine. Rowe and co-workers (11) claimed that the use of immature mice is less practical than the use of rats and no more accurate than other methods of assay. They found that four of their rat units (based upon the production of corpora lutea in rats 26 days old at the start of injection) are equivalent to one Katzman-Doisy (12) unit (mouse-vaginal smear). In a similar manner, they found that the rabbit ovulation unit of Friedman (13) is equivalent to 1 R.U. per kg. of body weight of rabbit.

The Chick Assay

Numerous workers have shown that the testes of the dove or immature pigeon will respond to the injection of pituitary extracts or to homeotransplants. Riddle and Polhemus (14) showed that the testis of the dove is not responsive to the injection of pregnancy urine preparations. Evans and Simpson (15) showed that the dove testis reacts to the injections of pregnant mare serum, but that it requires a higher number of rat units of this material than of their pituitary extracts to produce the same effect. Hamburger (16) observed that the injection of pregnant mare serum into young cockerels produced a rapid growth of the testes and comb of the injected animals. Byerly and Burrows (17) studied the response of the 1-day-old chick to pregnant mare serum. They found a straight line relationship when the logarithm of the testes weight was plotted against the logarithm of the dosage. They proposed the following chick unit: "a male chick unit of gonadotropic hormone be defined as that amount of material which when injected subcutaneously into a male chick during the first day after hatching will produce testes double the weight of control testes 72 hours after the time of injection." These authors found that about 10 R.U. (Cole and Saunders units) were required to double the weight of control testes when injections were made in the manner described.

SUMMARY

In the assay of unfractionated pituitary extracts, the mouse uterus is about 66 times as sensitive as the rat ovary, and about 10 times as sensitive as the chick testes. In the assay of pregnant mare serum preparation (gonadogen), the mouse uterus is about 60 times as sensitive as the rat ovary, and about 90 times as sensitive as the chick testes. In the assay of normal male urine preparation (prospermin), the mouse uterus is about 90 times as sensitive as the rat ovary, and about 55 times as sensitive as the rat uterus. The response of the chick testes to normal male urine is doubtful. The mouse uterus is about 30 times as sensitive as the rat ovary to menopause urine preparation (gamone) and about 6 times as sensitive as the rat uterus. The response of the chick testes to menopause urine is doubtful.

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COMPARATIVE STUDY ON THE AUGMENTATION OF THE ACTION OF GONADOTROPINS FROM VARIOUS SOURCES IN THE RAT, MOUSE AND CHICK^{1,2}

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MANY OBSERVATIONS (1, 2, 3) have been made which clearly show that the response of the immature female rat to gonadotropic material of glandular origin is complicated by augmenting effects as well as by antagonists. The purpose of this study is to investigate further the degree of augmentation brought about by various reagents with 4 types of gonadotropin preparations in 3 species and to attempt to throw more light on the mechanisms involved.

Assay methods. The rat method is similar to that used by Wallen-Lawrence (4). Female rats, 21 to 23 days of age, were injected subcutaneously once daily for 4 days and autopsied 48 hours after the final injection. The ovaries and uteri were dissected free from adhering tissues and fat. The uteri were trimmed by cutting through the utero-tubal junction and through the junction of the cervix and the uterus, and freed from fluid by pressing against paper toweling. The organs were weighed on a Roller-Smith torsion balance.

The mouse assays were conducted by the method of Hamburger. Immature female mice, weighing 6 to 8 gm., were injected twice daily for 3 days, and 48 hours after the last injection the ovaries and uteri were dissected and weighed.

The chick assays were conducted by injecting groups of about 20 1-day-old cockerels subcutaneously in the front of each leg twice daily for 5 days and weighing the testes 18 hours after the last injection. In certain of the experiments, the injection period was shortened to 2 days.

Gonadotropin preparations. The glandular product, prep. 137, was prepared by suspending acetone-dehydrated sheep pituitaries in 0.25% ammonium hydroxide for 24 hours, adjusting the pH to 5.0 with acetic acid and centrifuging off the gland residue. The active material was precipitated from the supernatant solution by adding 3 volumes of ethyl alcohol. The precipitate was centrifuged off and extracted with 0.25% acetic acid. This solution is called prep. 137. The pregnancy urine preparation was made by adsorbing the gonadotropic substance on benzoic acid and subsequently precipitating the active material by the addition of a large excess of acetone. Gonadogen is the Upjohn Company's preparation made from pregnant mare serum. Prospermin and gamone are the Squibb & Sons' preparations made from normal male and menopause urines respectively.

Effect of copper and zinc salts in the rat. The results in table 1 show that the addition of zinc or copper salts to sheep pituitary extracts produced an augmentation in

¹ These investigations were supported by a grant from Armour & Company.

² We are indebted to Dr. George F. Cartland of the Upjohn Company for the gonadogen, to Dr. J. A. Morrell of Squibb & Sons for the prospermin and gamone, to Dr. Edwin F. Pike of Armour & Company for the pituitary glands, and to Dr. G. H. A. Clowes of Eli Lilly Laboratories for the protamine used in these studies.

the response of the rat ovary, but when the extract and Cu or Zn salts were injected separately little or no augmentation was observed. The slight increase obtained when the Cu salts and the extract were injected separately may have been due to the slight mixing of the two solutions in the subcutaneous area after the injection. The addition of Cu and Zn salts to the chorionic substances, pregnancy urine and pregnant mare serum preparations, did not produce augmentation. The slight increase in the uterine weight may reflect a slight effect but since the uterine response is already well up on the uterine curve, it probably does not have any meaning. Addition of Zn salts to solutions of prospermin or gamone did not produce an augmentation. Hemin augmented the response of the rat ovary to a given dose of pituitary extract.

TABLE 1. THE EFFECT OF COPPER SALTS, ZINC SALTS, AND HEMIN ON THE ACTION OF VARIOUS GONADOTROPINS IN THE RAT

Gonadotropic substance	Injected together		Injected separately	
	Ovarian wt.	Uterine wt.	Ovarian wt.	Uterine wt.
	mg.	mg.	mg.	mg.
Prep. 137, 0.5 cc.	20.9	32.7		
Prep. 137, 0.5 cc.+1.6 mg. ZnCl_2	62.7	57.4	26.7	28.7
Prep. 137, 0.5 cc.+2.7 mg. CuSO_4	86.7	68.8	33.2	29.6
PU ¹ Prep. 99, 1.5 mg.	17.7	78.0		
PU ¹ Prep. 99, 1.5 mg.+2.7 mg. CuSO_4	24.2	97.9	14.3	65.2
Gonadogen, 0.5 R.U. ²	22.5	123.6		
Gonadogen, 0.5 R.U. ² +2.7 mg. CuSO_4	18.3	148.0	27.2	107.9
Prospermin, 5 Sq. R.U. ²	22.3	115.2		
Prospermin, 5 Sq. R.U. ² +2.1 mg. ZnAc.	28.3	110.2		
Gamone, 5 Sq. R.U. ²	23.6	111.5		
Gamone, 5 Sq. R.U. ² +2.1 mg. ZnAc	23.6	83.4		
Prep. 137, 1 cc.	27.8	26.9		
Prep. 137, 1 cc.+5 mg. hemin	36.2	40.3		

¹ PU refers to pregnancy urine preparation.

² R.U.=rat units as expressed by the manufacturer.

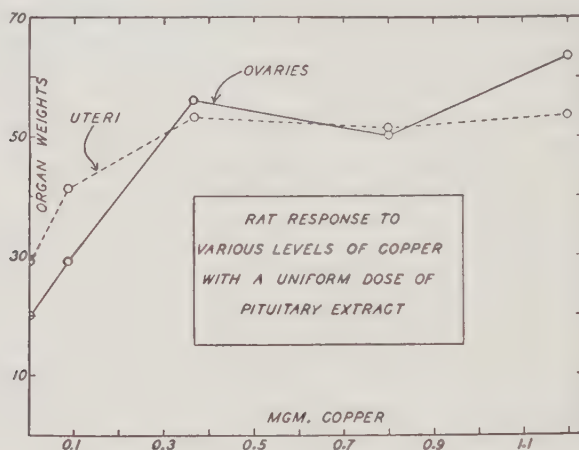
The results of our experiments (table 2) and those of Pfeiffer (16) indicate that the action of augmenting agents, such as Cu or Zn salts, is not due in any way to its action upon the animal's endogenous hormones.

TABLE 2. EFFECT OF COPPER AND ZINC SALTS UPON THE ENDOGENOUS HORMONES OF THE IMMATURE RAT

Injected material	Ovarian wt.	Uterine wt.
	mg.	mg.
0.9% NaCl	11.5	19.0
0.012 N CuSO_4	10.5	18.9
0.012 N ZnCl_2	10.1	16.6

The effect of increasing the concentration of Cu per hormone unit. Saunders and Cole (5) showed that the addition of varying amounts of zinc sulfate or of casein to a constant dose of pituitary extract produced increased ovarian weights in the rat with increased dosage. Their results were confirmed in our laboratory by adding various amounts of copper sulfate to a constant dose of extract (fig. 1). Ovarian weights increased with increasing amounts of salt added until a total dose of 1 mg. of CuSO_4 was reached. At this level, the response tended to plateau off. At about the same level, the toxic effects of the Cu salts are first noticeable by a loss of weight, loss of hair and the development of sores at the site of injection on the animals.

Effect of protamine in the rat. Fevold, Hisaw, Hellbaum and Hertz (2) precipitated their active material in the form of tannates and injected suspensions of their precipitates. They believed that the tannic acid formed a combination which retarded the rate of absorption of the hormone. McShan and Meyer (6) have pointed out that there is not always a correlation between the formation of insoluble precipitates and



the production of augmentation. Bates and Riddle (7) found that prolactin was precipitated from solution by the addition of Cu and that the supernatant solution was very potent with respect to gonadotropic and thyrotropic hormones. Their findings suggest that the augmentative power of Cu salts does not lie in the formation of an insoluble precipitate. Table 3 shows the results of an experiment in which a total dose of 2 mg. of protamine (Lilly) was combined with a total dose of 0.5 cc. of pituitary extract at various pH levels. When the solution was pH 6.5 or above, a flocculent precipitate occurred, but no augmentation was produced. No augmentation was produced at lower pH levels (table 3).

TABLE 3. EFFECT OF THE ADDITION OF PROTAMINE TO PITUITARY EXTRACTS IN THE IMMATURE RAT

No.	Total dose pituitary 137	Total dose protamine	How administered	pH	Ovarian wt.	Uterine wt:
	cc.	mg.			mg.	mg.
1	0.5	0.0	—	4.5	32.2	45.1
2	0.5	2.0	together	4.5	36.9	48.2
3	0.0	2.0	—	—	9.7	17.2
4	0.5	2.0	separately	4.5	32.3	45.8
5	0.5	0.0	—	4.5	31.0	33.1
6	0.5	2.0	together	6.8	20.9	32.3
7	0.5	2.0	together	10.8	25.8	48.9

When the solutions were injected separately they were injected subcutaneously at different sites.

Breneman (8) showed that the addition of a suspension of 0.25 mg. hemin to pituitary extract did not produce any augmentation. If sufficient pyridine was added to dissolve the hemin, augmentation did occur. McShan and Meyer (6) have suggested that this may be due to the formation of a hemochromogen-like compound. It is apparent that retarding of absorption by the formation of insoluble precipitates is probably not the only mode of action of an augmenting agent.

Effect of adrenalin on the gonadotropic response in the rat. Tannic acid (2), zinc

salts (1), merthiolate (9), casein (5), and other compounds have been reported to act by slowing down the rate of absorption and possibly reducing the toxicity. Adrenalin, by inducing local vasoconstriction and thus retarding the local absorption, prolongs the effects and reduces the toxicity of cocaine and similar local anesthetics. Hence adrenalin was added to pituitary extracts and gonadogen in order to retard the rates of absorption by local vasoconstriction. The effects of adrenalin upon these substances are shown in table 4.

TABLE 4. THE EFFECT ON THE RAT ASSAY OF THE ADDITION OF ADRENALIN TO GONADOTROPIC SUBSTANCE

No.	Gonadotropic substance	No. of daily injections	Addition	Ovarian wt.	Uterine wt.
				mg.	mg.
1	0.5 cc. pituitary 137	2	—	33.8	42.7
2	0.5 cc. pituitary 137	2	Adrenalin ¹	75.0	65.0
3	0.5 cc. pituitary 137	2	—	28.9	42.0
4	0.5 cc. pituitary 137	2	Adrenalin	26.9	35.9
5	1.0 cc. pituitary 137	2	—	62.9	48.0
6	1.0 cc. pituitary 137	2	Adrenalin	100.0	84.3
7	0.83 R.U. gonadogen	1	—	35.8	167.1
8	0.83 R.U. gonadogen	1	Adrenalin	30.6	149.1

¹ In 2, 6 and 8, the mixture of gonadotropin and adrenalin was injected subcutaneously. In 4, the adrenalin and pituitary extract 137 were injected separately. The concentration of adrenalin in 2, 6, and 8 was 1:10,000; in 4, an equivalent amount of 1:10,000 adrenalin was injected.

The addition of adrenalin to the extracts from sheep pituitary produces an augmentation in the ovarian and uterine responses in the rat. When the adrenalin and the extracts were injected separately, no augmentation was observed. No augmentation was observed when adrenalin was added to the pregnant mare serum preparation. Possibly this negative effect is due to the fact that normally the hormone is very slowly destroyed and excreted from the body.

Administration of the hormone by heart puncture. In order to test the hypothesis that the augmentation, produced by the addition of CuSO_4 and other substances is due to a delay in the rate of absorption, two aliquots of the pituitary extract, one with and the other without CuSO_4 , were each injected into rats subcutaneously

TABLE 5. ADMINISTRATION OF THE HORMONE BY HEART PUNCTURE IN THE IMMATURE RAT

Substances injected	Mode of injection	Ovarian wt.	Uterine wt.
		mg.	mg.
Pituitary 137+ CuSO_4	Subcutaneous	36.5	65.3
Pituitary 137+ CuSO_4	Heart puncture	15.5	22.9
Pituitary 137	Subcutaneous	15.2	21.8
Pituitary 137	Heart puncture	14.8	22.2

and directly into the heart. The results are shown in table 5. The preparation containing CuSO_4 when injected by heart puncture gave approximately the same results as when the solution without the CuSO_4 was injected either subcutaneously or by heart puncture. The same total dose with the addition of CuSO_4 when injected subcutaneously produced marked augmentation.

Effect of vasodilators and diuretics in the rat. Our results thus far indicate that the augmenting agents act by retarding the rate of absorption. The converse of the action of adrenalin was tried in order to see if a vasodilator would increase the rate of ab-

TABLE 6. EFFECT OF SODIUM NITRITE AND DIURETICS ON THE RAT RESPONSE TO PITUITARY EXTRACT

Total dose prep. 137	Addition	Ovarian wt.	Uterine wt.	Remarks
cc.		mg.	mg.	
1.5	—	28.5	41.3	1 injection per day
1.5	0.6 mg. NaNO ₂	31.0	32.6	1 injection per day
1.5	1.67 mg. theophyllin	26.7	56.0	1 injection per day
1.5	1.5 mg. theobromine sodium acetate	32.2	47.6	1 injection per day

sorption. The first vasodilatin used was sodium nitrite (table 6). This substance has only a very temporary vasodilating action so the negative results are not unexpected. Isomannide dinitrate³ which has been shown to have a more prolonged vasodilating action in the dog (10) was used in the later experiments. The action of the isomannide dinitrate produced an increase in the response to a given dose of hormone rather than the expected decrease. This may be due to a general vasodilation producing a fall in blood pressure and consequent retention of the hormone in the blood stream (see table 7).

TABLE 7. EFFECT OF ISOMANNIDE DINITRATE UPON THE RAT RESPONSE TO PITUITARY EXTRACT

Total dose prep. 137	Total dose ¹ vasodilator	Ovarian wt.	Uterine wt.	Remarks
cc.	cc.	mg.	mg.	
1.0	—	44.9	49.3	Injected subcutaneously
1.0	0.015	42.9	53.1	Injected subcutaneously
1.0	0.03	68.1	51.6	Injected subcutaneously
1.0	0.06	61.9	57.5	Injected subcutaneously
1.0	0.16	54.4	56.5	Injected subcutaneously
1.0	0.32	48.0	49.8	Injected subcutaneously
1.0	2.0	55.2	46.0	Isomannide made fresh daily, injected subcutaneously
1.0	8.0	54.8	56.2	Isomannide made fresh daily, given orally, stomach tube.

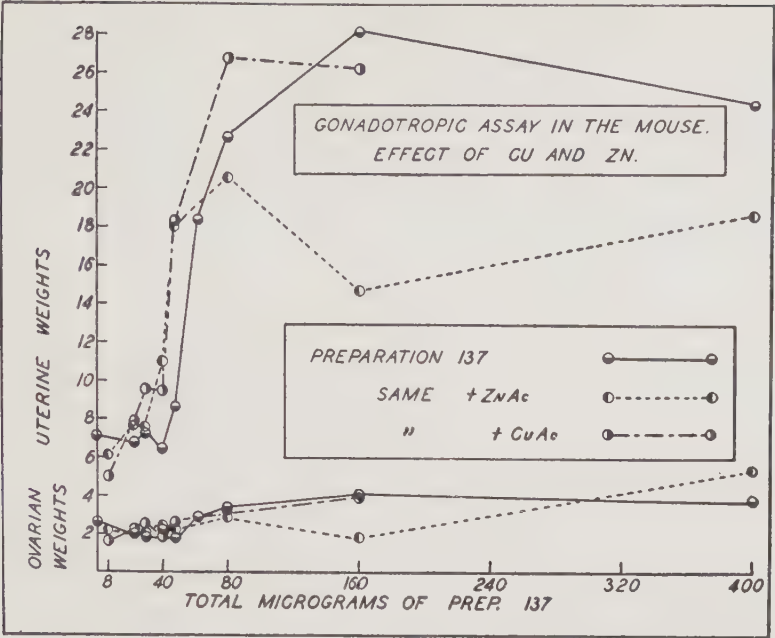
¹ The isomannide dinitrate was given as a 1:1000 solution. The solutions were injected twice daily for a period of 4 days and the animals autopsied about 40 hours after the final injection.

The effect of the addition of diuretics such as theophyllin and theobromine to the extract was studied (table 6). Theophyllin or theobromine had no effect on the assay. However, the diuretic actions of theophyllin and theobromine are not very marked in the rat (11). This may account for the failure to remove the hormone more rapidly from the circulation of the rat through the kidneys.

Attempts to augment the gonadotropin assay in the mouse. L. C. Maxwell (1) claims that the addition of zinc sulfate to minimal dosages of pituitary extracts exerts a strong estrogenic action on the rat uterus, but often without detectable morphological changes in the ovaries. Hamburger (12) and also Levin (13) have shown that the mouse uterus is very sensitive to substances which stimulate the ovaries to secrete estrogens, hence we were interested to study the effect of Zn, Cu, and adrenalin on the gonadotropic response in the immature mouse.

Figure 2 shows the effect of the addition of Zn and Cu salts upon the mouse assay of preparation 137. At the higher levels the Cu salts caused death. There is a slight

³ We wish to acknowledge our appreciation to Professor E. M. K. Geiling for this suggestion and to Dr. J. C. Krantz for the preparation used.



Shift of the uterine weight curves toward the left but no effect on the ovarian weight. No gross structural changes were observed in the ovaries of the mice. The negative effect of the addition of adrenalin to pituitary extracts upon the mouse assay is shown in table 8.

TABLE 8. EFFECT OF ADRENALIN ON THE GONADOTROPIC RESPONSE IN THE MOUSE

Gonadotropic substance	Body wt.	Ovarian wt.	Uterine wt.	No. of animals
	gm.	mg.	mg.	
0.1 cc. pituitary 137	9	2.3	16.2	7
0.1 cc. pituitary 137 adrenalin, 1:10,000	10	2.6	16.1	5
Controls	7	2.5	7.1	5

Levin and Tyndale (13) have shown that the mouse ovarian weight is a very poor gonadotropic criterion and even with the use of large numbers of animals errors of less than 100% are not avoidable. They also state that castrate urine extracts are less likely to produce luteinization in the mouse than in the rat. Engle (14), however, claims that with pituitary extracts, rats tend to show less luteinization than mice.

Attempts at augmentation in the chick. Breneman (8) reported that the addition of augmenting agents to pituitary extracts produced an increase in the chick testis weight over that expected from a given amount of pituitary extract. The augmentation reported was, however, of a very low order, i.e., chlorophyll produced an increase of 1.6 mg. and auxilin produced an increase of 2.0 mg. in average testis weights. It was felt advisable to repeat the studies with better established augmenting agents.

Our first series of chicks was injected twice daily for 5 days and autopsied about 18 hours after the final injection. The second series was injected twice daily for 2 days and autopsied 18 hours after the final injection. The results are shown in table 9.

Preparations containing Zn salts were made up in such a manner that the concentration of the Zn was 0.012 N. The adrenalin solutions were 1:10,000. Prep. 169

TABLE 9. EFFECT OF AUGMENTING AGENTS ON THE CHICK ASSAY

Total dose	Addition	No. of chicks	Average testes and range	Standard deviation
			mg.	
<i>5-Day Injection Series</i>				
Prep. 169, 10 mg. sheep ¹ D.P.	—	20	14.7 7.8-22.7	3.98
Prep. 169, 10 mg. D.P.	ZnAc	8	16.1 6.4-31.2	8.24
Prep. 169, 10 mg. D.P.	Adrenalin	11	14.7 9.1-27.4	5.58
26-S (Beef ext.), 1.0 mg	—	38	12.7 7.1-24.0	4.64
26-S (Beef ext.), 1.0 mg.	Adrenalin	14	12.1 7.7-28.6	5.47
Controls	—	52	9.9 4.0-21.0	3.80
6.0 mg. PU 92 ¹	—	10	11.3 7.9-16.9	3.36
<i>2-Day Injection Series</i>				
Saline controls	—	17	9.9 3.7-16.4	2.72
Prep. 169, 20 mg. sheep D.P.	—	18	15.8 8.3-27.7	5.08
Prep. 169, 20 mg. D.P.	ZnAc	20	19.6 7.3-37.6	7.97
Prep. 169, 20 mg. D.P.	Adrenalin	16	13.3 8.8-26.7	4.21
Prep. 169, 20 mg. D.P.	1.5 mg. P.U. 318	19	19.1 7.7-38.7	7.50
Uninjected controls	—	9	8.2 4.0-18.2	4.42

¹ PU refers to pregnancy urine preparation. 6.0 mg. PU 92 is equivalent to 17.5 cc. urine; 1.5 mg. PU 318 is equivalent to 20.0 cc. urine.

$$\text{Standard deviation} = \sqrt{\frac{\sum d^2}{N-1}}$$

where d = the difference between the individual testes weights and the average testes weight, N = the number of individuals.

was prepared from sheep pituitary acetone powder and the dosage is expressed in terms of the equivalent of desiccated powder. The beef extract was prepared by extracting desiccated beef powder with dilute acetic acid.

In the 5-day series of injections, the Zn salts produced a slight increase in the average weight of the chick testes. However, the number of birds in this group was small and there was a wide variation in response to the material. In the 2-day series of injections, the addition of either Zn acetate or prolan produced an increase in the

average testes weight of about 3.5 mg. The standard deviation, even with a large series of animals was high. The addition of adrenalin to the extract using either the 2- or 5-day series of injections did not produce any augmentation. Because of the high standard deviation, we are not certain that Zn salts or prolan have any augmentative action in the 1-day-old chick. The wide variation in response to a given dose makes it imperative that one use a large number of animals on a given level.

Since it is possible to measure the thyrotropic activity of a preparation by the chick method it is interesting to note that no augmentation of the thyrotropic hormone was observed when Zn salts or adrenalin were added to the pituitary extracts.

DISCUSSION

The mechanism of the augmentation reaction has been believed to be due to a slower rate of absorption of the hormone with a consequent lowering of its rate of excretion and/or destruction. Tannic acid (2), Zn salts (1), Cu salts (3), merthiolate (9), egg albumin (5), heme (6), yeast extract (3), yeast ash (3), and a group of other substances (15) have been reported as effective augmenting agents. Fevold, Hisaw and Greep (3) claim that the action of Cu salts is not simply due to a delayed rate of absorption, but that Cu acts possibly by 'catalyzing' the synergistic action of the follicle stimulating and luteinizing hormones. They showed that the intravenous administration of copper acetate (10 mg.) produces ovulation in the rabbit. Saunders and Cole (5) confirmed Fevold and coworkers' observation (3) that Cu produces ovulation in the rabbit but that Zn salts, casein, or egg albumin do not do so. Fevold and coworkers (3) claimed that Zn salts augment FSH and FSH plus LH in normal and hypophysectomized rats, whereas Cu salts augment FSH plus LH, but do not enhance FSH alone in hypophysectomized rats. According to them, Cu and Zn salts augment both FSH and FSH plus LH in the normal immature female rat.

Pfeiffer (16) showed that the injection of CuSO_4 into constant estrous rats produced only a slight disturbance of the estrous smear. He attributed this to the toxic action of the CuSO_4 and concluded that the salt had no effect on the animal's endogenous hormones. He further showed that the injection of Cu salts did not produce excessive luteinization of the ovaries of immature rats. Copper sulfate injected into normal and castrate males bearing ovarian grafts in the anterior chamber of the eye, produced no augmentation on the grafts detectable during the experiment or at autopsy. He pointed out that the negative results obtained may be due to the small amount of the endogenous hormones in the blood stream, to the rapid elimination of the salt or extract, or to the necessity of the formation of a protein-salt complex.

Maxwell (1) showed that the addition of Zn salts to a pituitary extract resulted in an increased response to the extract in the rat, but that by distributing the dosage without the addition of Zn salts, he could obtain nearly the same effect. Maxwell believed that the Zn salts act by delaying the rate of absorption of the extract.

McShan and Meyer (6) showed that whole blood, erythrocytes, hemoglobin and heme augment the response in the rat to an extract of sheep pituitary glands. They attributed the augmentation to the breakdown of these substances into heme. Inorganic iron showed no enhancing activity. The authors point out the possibility of the formation of a hemochromogen-like compound which would be more active than the gonadotropic complex. Krebs (17) showed that heme is a poor 'catalyst' and that when heme is combined with certain nitrogenous compounds to form hemochromogens, the catalytic effect of the heme is dependent upon the group with which the heme is combined. The authors feel that the action cannot be attributed to a delay in the rate of absorption since there was no insoluble precipitate formed

when heme was added to the extract. They pointed out that the formation of insoluble precipitates and augmentation are not necessarily associated. This was also demonstrated in this laboratory when protamine was added to pituitary extracts with the formation of an insoluble precipitate, but augmentation was not produced.

Cassida (18) showed that the augmentative action of beef blood is due to the formed elements rather than to the plasma. Kraatz (19) claims that leucocytes possess an augmenting action. Preliminary experiments by him indicated that there is a rise in eosinophil content after the injection of Zn salts, either alone or in combination with FSH, into immature females, while injections of the hormone alone tend to lower the eosinophil number. Kraatz suggested that the number of circulating eosinophils may be an indication of the luteinizing activity and that these cells may play a rôle in the control and transport of the luteinizing hormone.

In our studies, we have confirmed the work of numerous investigators who showed that Cu salts, Zn salts, and heme augment pituitary extracts in the assay based upon the rat ovary. The general conception is that such substances act by delaying the rate of absorption. That a delay in the rate of absorption will produce an augmentation is shown by our adrenalin and heart puncture experiments. That all gonadotropins do not react in a similar manner is evident from the work on extracts of pregnancy urine, normal male urine, castrate urine and pregnant mare serum. Neither do all species react, at least to the same degree, to pituitary extracts containing augmenting substances. The fact that agents which are very effective in producing an augmentation of pituitary extracts in the immature female rat have little, if any, effect in other species such as the mouse or chick suggests that the mechanism may not be entirely a delay in the rate of absorption. This is further emphasized by the fact that substances capable of delaying the rate of absorption do not augment other gonadotropins than pituitary extracts. The results of our experiment and those of Saunders and Cole (5) show that there is an optimum amount of augmenting agent which should be added to a given dose of extract in order to obtain the best response. Since many substances augment pituitary preparations and other substances or extracts antagonize the response to pituitary preparations, it is evident that such disturbing factors must not be ignored in assaying gonadotropin preparations.

SUMMARY

The subcutaneous injection into immature female rats of mixtures of Zn salts, or adrenalin with pituitary extracts cause a marked augmentation in ovarian and uterine responses. When injected separately, no such augmentation is observed. Preparations from pregnancy urine, pregnant mare serum (gonadogen), normal male urine (pro-permin) and menopause urine (gamone) are not augmented by Zn salts, adrenalin, or Cu salts in the immature female rat. Copper and Zn salts do not augment the animal's endogenous hormones. Heart injection experiments gave additional evidence that in the rat the Cu produces its augmentation in part by delaying the rate of absorption of the hormone. Sodium nitrite, theophyllin and theobromine have no effect on the rat ovarian response to pituitary extracts. Isomannide dinitrite augments the rat ovary response to pituitary extracts. Zinc and Cu salts, when added to pituitary extracts, exert a slight augmentation in the mouse uterine response. Zinc and Cu salts, as well as adrenalin, fail to augment the pituitary extract when the weight of the mouse ovary is the criterion of assay. Zinc salts and adrenalin do not augment the chick testis response to pituitary extracts in a 5 day period of assay. Adrenalin in a 2-day test period produces no augmentation.

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